"A Sugar Bacterium." By H. MARSHALL WARD, F.R.S., and J. REYNOLDS GREEN, F.R.S. Received March 2,—Read March 9, 1899.

In the 'Annals of Botany' for 1897,* one of us published a short note on a curious organism—or rather association of organisms—obtained in Paris, and said to have come from Madagascar, where it occurs as "an excrescence on the sugar-cane."

It consists of a bacterium associated with at least one yeast, and grows in saccharine solutions, producing clumps so like the ginger-beer plant† that the assumption seemed warranted that we had here a symbiosis of the same kind as that proved to occur there.

Moreover, the general course of events in the use of this body, which is employed to make a fermented effervescing drink from common brown sugar in water, points to the same conclusion.

In moderately strong solutions containing 15 to 20 per cent. of common sugar in water, the clumps referred to induce a powerful fermentation, resulting in the liberation of relatively enormous quantities of carbon dioxide and some acid, the saccharine liquid being thus converted into a not unpleasant acid drink, with some resemblance to lemonade or ginger-beer.

From the fact that this fermentation occurs rapidly when the corked flask is entirely filled with the recently boiled sugar solution, infected with a few clumps of the organism, it is clear that oxygen is not necessary in any quantity.

This conclusion is also confirmed by the observation that if a bottle of soda-water is opened, and a handful of sugar added with a few of the clumps, and at once corked and wired, the pressure of the carbon dioxide liberated during the active fermentation which at once ensues becomes so great in three or four days at 22° C., as to cause danger of bursting the flask; and if a manometer tube with mercury is attached, as described in the paper above referred to,‡ the bubbles of gas pressed out come off steadily for many days, or even weeks, at ordinary temperatures, until no more sugar is left.

The general resemblances to the well known kephir, also referred to in the previous paper, led one of us to repeat the above experiments with sugar and milk, instead of soda-water, with the result that carbon dioxide came off as before until all the sugar had disappeared, the milk meanwhile undergoing coagulation into clots, but since these clots remained unaltered for weeks or months, this experiment suggests

* Marshall Ward, "On the Ginger-beer Plant," 'Annals of Botany,' 1897, vol. ii, p. 341.

† 'Phil. Trans.,' B, 1892, pp. 125–197.

‡ *Loc. cit.*, p. 137.

that the organism—unlike kephir—does not ferment the milk itself, but only the added sugar, and that the clots are simply due to the acids liberated as the sugar is destroyed, a conclusion fully borne out by subsequent investigations.

A preliminary experiment with the ginger-beer plant showed that it also behaves in the same way as regards milk; the fermentation only occurs if sugar be added, and lasts only so long as any sugar remains.

In the cases both of the present organism and of the ginger-beer plant, if a clump be placed in sterilised beer wort, the resulting fermentation gives a frothing liquid with a beer-like taste and smell, and a rapid deposit of yeast occurs. This primary fermentation is very soon finished, and it is abundantly proved by the experiments that this medium favours the yeast—or one of the yeasts—in the clumps, so that we may regard this fermentation as merely a particular case of an impure alcoholic fermentation, such as is got in ordinary brewing.

During these preliminary trials, and with the object of testing whether acidification of the liquid from the first would affect the matter, it happened to one of us to select lemon-juice as the medium in one case, partly to acidify the medium, and partly to add vegetable matter other than sugar. The result was somewhat astonishing. The mixture of soda-water, sugar, and lemon passed into violent fermentation in three days, and carbon dioxide came off abundantly, under a pressure of about 12 inches of mercury, and continued to do for many days.

Such a flask started on April 9, 1897, was evolving gas actively on the 12th; this went on without any apparent diminution until May 24, and even on June 24 gas was still coming off, though now under less pressure. No sugar had been added in the interval, and the flask, still unopened, remained on a side bench in the laboratory until January 18, 1898. On that date it was opened and examined. It still stood at a pressure of 6 inches of mercury, and gave off gas as soon as the pressure was reduced. The microscopic examination showed that here, again, as in the beer wort, the medium had favoured one of the yeasts, and although the bacterium was discoverable, it was in abeyance, and the compound organism as a whole was not increasing.

In another similar case the flask was started on May 28, 1897, and the pressure of the gas evolved was still supporting nearly 12 inches of mercury on April 23, 1898, and again only yeast predominated in the deposit, and examination showed this to be fully alive; it at once renewed its activity when placed in sugar solutions.

These preliminary experiments will suffice to show that we have in this compound of associated organisms an agent or agents capable of setting up very active fermentation in various saccharine liquids, such as ordinary sugar and water, or soda-water, beer wort, milk, and sugar, or an infusion of vegetable substance, such as lemon pulp, and it is clear that the fermentation, though differing in details in each case,

always results in the destruction of the sugar, and the production of enormous quantities of carbon dioxide. Obviously, also, these fermentations are anaërobic.

In order to put this last point beyond all cavil, however, we placed a clump of the compound organism into a mixture of a 15 per cent. solution of sugar to which 10 per cent. gelatine had been added, and kept the tubes at 30° C., while the air was pumped out from the fluid mass and pure carbon dioxide allowed to filter in, and this process was repeated four or five times, and the de-oxygenated gelatine, still in an atmosphere of carbon dioxide, was then allowed to set. In a fortnight the solid gelatine in all these tubes had visible submerged colonies throughout the mass, and examination showed these to consist of the bacterium and yeast found in the original clumps.

Similarly, streak cultures on the same sugar-gelatine medium, grew normally on the sloped surface in tubes filled with carbon dioxide, and in these again were observed the same bacterium and the same yeast as had been found predominating in the original clumps.

These cultures—still preliminary in nature and only dealing with the composite organisms of the clumps as a whole—suggested an obvious method for separating at least this prominent bacterium and yeast from the clumps.

Sugar-gelatine, made as before, was infected with a small piece of a clump, rubbed up by a platinum loop in sterile water, and plates made in the ordinary way in Petri dishes; the dishes were then placed under a receiver, attached to the pump and exhausted, and then filled with carbon dioxide, with proper precautions as to the purity of the gas, filtration through cotton-wool plugs, &c., and the cultures put aside in an atmosphere of carbon dioxide at the ordinary temperature. In a week the solid gelatine showed two kinds of colonies, one consisting entirely of a yeast, the other of a bacterium, and closer investigation showed them to be identical with the prevailing yeast and bacterium in the original clumps.

Repeated plate-cultures made in this way gave consistently the same results, and there was no room for doubt that these are the two essential organisms of the clumps, though they are not the only species found in the original material, there being at least one other yeast-like organism, so common that for some time it was thought it must play an essential part. Since this latter—and certain much rarer forms occasionally found—will not grow in the atmosphere of carbon dioxide, however, there can be little doubt that the two anaërobic microbes isolated by the above process are the essential constituents in the fermentations referred to.

Their further separation by means of repeated plate-cultures, as above, was comparatively easy, the yeast especially being readily picked out and further cultivated in sugar-gelatine tubes.

As it is not at present proposed to deal with the yeast, which appears to be a mere variety of *S. cerevisiæ*, we pass on to the cultures of the bacterium only.

On repeating the plate cultures exactly as before, except the exhaustion and filling with carbon dioxide, it was found that mixtures of the yeast and bacillus grew as well in air as in carbon dioxide. At first it seemed possible that this was because the yeast rapidly consumed the oxygen and so prepared an oxygen-free atmosphere for the bacterium, but further experiments proved that this is not necessary, and that both yeast and bacterium can be grown in air as well as in carbon dioxide or in hydrogen.

The appearance of the separated bacterium on the sugar-gelatine plates is that of circular, raised, dome-shaped, watery-looking colonies, stiff, like a firm jelly, and lifting as a whole on the needle. Each colony is, in fact, a firm zoogloœa composed of short rodlets in pairs or chains, the cell-walls of which are so swollen as to furnish the zoogloœa jelly. The average size of these rodlets is 2—3 μ long by 1 μ thick, though much longer rods and filaments occur in other media.

Having once obtained the organism in pure culture, it was, of course, easy to test its behaviour on various media. It is unnecessary to enumerate all the media tried, or to give details of the cultures, which amount to several hundreds; enough that all ordinary media employed by bacteriologists were tried, as well as a long series of special ones devised to meet the suggestions which arose during the course of the investigation.

A striking fact comes out on surveying these cultures, namely, this Schizomycete practically refuses to grow in or on any pabulum devoid of sugar, and, further, only certain sugars are capable of supplying it with its necessary food. No growth at any temperature could be obtained in normal gelatine-peptone media, or in broth, milk, or other animal extracts, *e.g.*, serum-agar, such as is used by the animal pathologists.

Gelatine Cultures.

Gelatine, 10 per cent., added to "black sugar" solution* 15 per cent. is a capital medium for cultures at 18° C., or thereabouts, and in air, in hydrogen, or in carbon dioxide, the bacterium formed prominent domed colonies looking like drops of stiff gum or gelatine.

This black-sugar gelatine was also used for cultures *in vacuo*. Plates kept under a receiver permanently attached to the pump, going day and night continuously for a week, showed traces of colonies in eight days, and in fourteen days the colonies in the exhausted receiver were

* This "black sugar" is a very dark coarse Demerara sugar and probably contains considerable quantities of mineral and other matter.

as well developed as those in control plates in air at the same (low) temperature.

Streak cultures on black-sugar gelatine with yeast extract developed very rapidly at and near 18° C. Instead of a spreading slimy mass as on agar, these formed dense gelatinous, almost brittle, tear-like drops and streaks standing a millimetre or more high, and curling up off the surface of the gelatine in a most remarkable and characteristic manner.

On saccharonal-yeast-extract-gelatine similar raised streaks were obtained, but less luxuriant than on brown sugar; possibly because the temperature was lower, or only 10 per cent. saccharose was employed, or from lack of minerals.

Beer-wort gelatine gave very slight indications of growth, soon stopping, and never attaining anything like these dimensions, the mere traces observed being probably due to saccharose in the wort.

Certain mineral solutions—*e.g.*, Kleb's solution—appeared to inhibit the growth in the black sugar and gelatine.

We have here gathered together the principal facts of its behaviour on gelatine media, because they seem to bring out clearly that the gelatine itself has little or no part to play in the nourishment of the bacillus: mere traces of superficial liquefaction occur, and several experiments show that it is of no slight importance what is added to the gelatine, *e.g.*, the surprising results of beer-wort gelatine.

Agar.

Agar (2 per cent.) made up with black sugar and yeast water rapidly formed large slimy blister-like colonies in four days, both at 25° C. and at 19° C. The contrast between these large, slimy, flat, and extended colonies and the small, raised, dome-like, stiff ones, on gelatine was very marked.

Streak cultures on black sugar agar, with yeast extract, grew rapidly as a dull, honey-like slime, spreading all over the surface in two days at 16—18° and at 23—25° C., as well as at 36—37° C.

Agar made up with peptone, &c., was of no use whatever, nor was potato-agar. Even peptone-agar with black sugar proved unsuitable.

Similarly unsuitable was agar made up with yeast extract and saccharose, which had been filtered through porcelain, though a pale dotted streak formed at first at 25, 27, and 31° C.

Agar (2 per cent.) made up with 10 per cent. saccharose and yeast extract not filtered through porcelain, on the other hand, was an excellent medium. In twenty-four hours at 30—31° C., a rapidly growing slimy streak had formed, consisting of filaments up to 60 μ long and more, breaking up into segments of all lengths down to 1·5 μ by 1 μ. These showed no sheaths—they had turned slimy—stained

well in gentian violet by Gram's method, and were non-motile. At 23° and 20° C. the growth was similar but slower.

Wort-agar, however, was unsuitable, cultures parallel with the above, showing very slight growths at any temperature; results quite conformable with previous experience.

Here, again, we must conclude that the agar is of little or no importance, except as a support. The significance of the failure on agar, to which porcelain-filtered yeast extract and saccharose was added, appears significant, and we shall return to this in order to discuss what happens to the yeast extract and sugar when this mode of sterilisation alone is relied on.

Beer Wort.

In beer wort (unhopped) the bacterium grows fairly well at first, rapid turbidity following the infection, and a dirty yellowish deposit soon falls, consisting of the flocculent bacterial masses carrying down colouring matter; but the liquid is not viscous, and the deposit scarcely slimy, and growth soon ceases.

When we reflect that beer wort—*i.e.*, malt extract—is usually an excellent medium for the growth of fungi, it is somewhat surprising that this sugar-loving bacterium should do so badly in it. Thinking that the failure might be owing to the kind of sugar in beer wort being unsuitable, we tried adding saccharose, but no obvious improvement was effected. We have seen (p. 69, above) that beer wort and gelatine gave poor results, and we concluded provisionally that some unfavourable substance occurs in wort.

Struck by the success of the preliminary trials with cane sugar, and remembering the alleged original habitat of the organism, it was determined to try the effect of beet: this was done not only because beet is a well-known source of cane sugar, but also because one of us had previously observed in a certain beet disease, that a bacterium very similar to the one under investigation spreads in the tissues of the sugar-beet, and is connected in some way with the disease itself.

Beet Extract.

Cold water extract of crushed sugar-beet was found at an early stage of the work to be a favourable medium. In tubes at 25° C., cultures in carbon dioxide rapidly become turbid, and on the third day, a dense slimy zoogloea-like deposit had fallen to the bottom carrying with it the colouring matters, and containing embedded bacteria. The same at 15° C. in carbon dioxide, the growth being simply somewhat slower.

Parallel cultures—from the same tubes—in broth, gelatine, or agar, devoid of sugars, showed no growth either in air or in carbon dioxide, at 15° or at 25° C.

Tubes of raw beetroot, infected with the gelatinous bacterial clumps, showed evident sinking into the tissues in twenty-four to forty-eight hours at 18°, 25°, and 31°, and in six days the depressed area showed collapsed and browned cells under the microscope. A curious white zone surrounded the discoloured patches. The impression gained was that the bacterium draws out the sugary sap and thrives on it: no proof of direct entry into the cell walls could be obtained.*

Cooked, *i.e.*, sterilised, beet gave a mere wet patch at first, but in a week raised gelatinous lumps of the typical kind were formed.

Here, then, we appeared to have proof that it is really the cane sugar which decides the success or otherwise of the cultures of this gelatinous bacterium, and we entered on what proved to be a very long series of trials with various kinds of sugars to decide this.

Trials with Sugars.

A series of preliminary experiments in which cane-sugar, glucose, and milk-sugar were employed, made up in various ways, soon showed that this bacterium grows far better in cane-sugar than in the others, and better in solutions made up with yeast-water than in such containing mineral salts, asparagin, tartrates, &c.

We, therefore, made series of parallel cultures at various temperatures, in air and in carbon dioxide, and all infected from the same tube, using the following sugars and solutions:—Levulose, pure glucose, cane-sugar, saccharon, lactose, maltose, dextrin, and a body known in Grübler's catalogue as "dextrin-zucker-lösung." The sugars were all made up in 10 per cent. solutions, and definite quantities—equal in each case—of the other ingredients added.

Summing up the results of numerous experiments, it was found that no growth occurred in any medium at temperatures of 35° C. and upwards, except in the case of certain agar cultures, where rapid growth occurred at and near 37° C. for a few days only.

Cane-Sugar.

The most striking results were obtained in all cases with cane-sugar, especially in a very pure re-crystallised form labelled "Saccharon," though we also employed ordinary lump-sugar, and a coarse dark brown moist sugar known locally as "Black sugar."

Made up as Mayer's solution, the brown sugar rapidly became turbid and viscous, and a dense gelatinous deposit of bacteria formed below

* Attempts at infection were made in view of the alleged connection between bacteria and certain diseases of beet and sugar-cane. See, for instance, 'Kew Bulletin,' No. 85, January, 1894, p. 1, and 'Zeitschr. f. Pflanzenkrankh.,' 1897, No. 7, p. 65.

the surface. This occurred even in tubes to which a few drops of absolute alcohol were added—a result not obtainable with dextrin-Mayer.

Far better growth was got with cane-sugar and yeast-extract however.

The best growth was got with saccharon (10 per cent.) and yeast extract, where at all temperatures from 16—27·5° to 31° C., the liquid became opalescent and viscous in two or three days, and deposited the typical gelatinous zoogloœa at the bottom of the tubes.

The results so far show that only the various forms of saccharose and beet extract (containing this sugar) afford any pronounced growth of this bacterium, the best results being got with pure saccharon and yeast extract, as indicated by the rapid turbidity and viscosity and the clump of gelatinous deposit. Since other sugars seem quite unsuitable, or only induced slight growths expressed as temporary turbidity and flocculent deposits, it may probably be assumed that in "dextrin-zucker"* and in "glucose" solutions, where indication of the viscosity and gelatinous deposit occur, traces of saccharose were contained.

Glucose.

Pure glucose and yeast-water encouraged an excellent growth at first, the liquid becoming turbid and a flocculent deposit settling down in a few days. No signs of viscosity appeared, however, and the deposit was quite loose and easily shaken up. The fairly abundant flocculent growth at first led to the expectation that prolonged cultures, or cultivation at different temperatures, might result in the development of the typical sliminess and viscosity, but repeated attempts show that such is not the case. This sugar did not appear to injure the bacterium, for the deposit was alive after three weeks.

Nor would it grow on or in gelatine made up with glucose. The slight growth obtained in certain cases where commercial glucose was used may have been due to admixtures. The total results show that glucose is not a favourable medium.

Levulose (*Fructose*).

Solutions of levulose prepared as the other solutions became slightly turbid in three or four days, and then the bacteria deposited as a very thin layer, no trace of slime or gelatinous matrix being formed, and the impression resulted that either traces of some other sugar must have sufficed for what activity was evinced in ordinary glucose solutions, because no growth worth mentioning compared with the preceding had occurred, or levulose is to a slight extent a food for the organism.

* This proved to be true of this medium; it consists of dextrin and cane sugar with a little alcohol.

Similar tubes placed in a vacuum also showed no growth beyond the formation of the flocculent deposit, hardly slimy, and easily shaken up into the clear supernatant liquid—a point of constrast of some importance, for in successful cultures in cane-sugar the viscosity of the liquid above is so great that it is very difficult to shake up the deposit, which is also dense and gelatinous.

Repetitions of these cultures gave the same results—a rapid development of a very slight turbidity and formation of a small non-gelatinous deposit which falls and leaves the liquid clear.

Some attempts were made to see if varying the strengths and composition of the levulose solutions would affect the matter—*e.g.*, Mayer's solution made with levulose gave little or no signs of growth at all with the bacterium, though it proved a splendid medium for the yeast.

Curiously enough, a mixture of beet extract and this levulose-Mayer's solution, though it encouraged abundant growth of the bacillus, rapidly becoming turbid and then clearing as the deposit fell, showed no viscosity nor was the deposit slimy, as occurs in beet extract alone.

Even made up with yeast-extract, no viscosity could be obtained: nothing beyond the slight flocculence, and it was clear that levulose is at best a poor food material for this bacillus.

Mixed Sugars.

Proceeding from the observation that the bacterium undoubtedly inverts saccharose, cultures were made as follows:—Dextrose-yeast-extract and levulose-yeast-extract were mixed, and infected with the bacterium: an excellent growth of the organism resulted, at both 32° and 24° C., but the deposit which resulted, consisted entirely of the non-sheathed bacterium in flocculent masses, and it remains a puzzle why the sheaths are formed in saccharose and not in the two sugars resulting from its inversion. The only conclusion seems to be that proportion of constituents has something to do with the matter.

"*Dextrin-Zucker-Lösung.*"

Under the above name, Grübler supplies a syrupy sugar in which the microscope shows delicate needle crystals, and—merely in the spirit of trying all experiments—this was tried with the following results:—

Made up with yeast water as a 10 per cent. or 15 per cent. solution, the bacterium slowly formed a large slimy deposit in from five to ten or twelve days at 15° and 23°, but not at 35°, in which rods and chains existed in the colourless matrix. The rods measured $1{\cdot}5\mu \times 1\mu$ to $3\mu \times 1\mu$,

but filaments up to 20 μ long were found. The growth resulted in a curious opalescent change in the liquid above the whitish dense deposit, and it became markedly viscous so as to draw out into strings.

Made up in Mayer's solution also, the same viscidity and gelatinous growth occurred, and this both in air and in hydrogen. We have since learnt that this syrup consists of dextrin, cane-sugar, and a little alcohol.

Dextrin.

The difficulty as to the nature of "Dextrin-Zucker" referred to above, led us to try dextrin itself, and owing to the kindness of Dr. Ruhremann some very pure material was to hand. With Mayer's solution several experiments demonstrated that no obvious growth occurred, and it was clear that this dextrin is not the same for nutritive purposes as the "Dextrin-Zucker" used previously.

Nor was dextrin made up with yeast-water of any use, though occasionally very slight indications of growth occurred during the first twenty-four hours, but no viscosity or sheathed bacteria formed.

Malto-dextrin.

Owing to the kindness of Mr. Ling, we were furnished with some prepared malto-dextrin, but neither as Mayer's solution, nor made up with yeast extract, was this sugar of any use as a medium for the growth of this bacterium.

Maltose.

This sugar, made up as Mayer's solution, was found unsuited for the bacterium, either at high or low temperatures. Nor was it more successful made up with yeast extract; a faint turbidity and flocculence occurred, but no trace of viscosity at 25° occurred in four days.

Milk-Sugar.

Milk-sugar, whether made up with yeast-water, Mayer's solution, asparagin, or peptone, proved quite useless as a food for the bacterium: no signs of growth appeared in the solutions at all, at high or low temperatures, in air, or in carbon dioxide. The organism was dormant only, however.

Soluble Starch.

It seemed worth while, in view of the gelatinous nature of vigorous growths, to see how the bacterium would behave towards soluble starch, but in no case could any evidence of growth be obtained in solutions containing 2 per cent. of soluble starch made up with Mayer's

solution, or with yeast-water. The bacteria lay dormant only at the bottom, as experiments showed.

It is evident from the foregoing that of all the sugars tried, saccharose is the one which favours the growth of the bacterium, but even in saccharon the growth is distinctly favoured by the addition of yeast extract. Some experiments were consequently started to test the effect of the yeast extract.

Raw Yeast-Water.

Fresh yeast, squeezed and drained, and then ground up with kieselguhr and extracted with water, was allowed to stand all night and filtered through porcelain. Employed alone it was of no use as a medium for the growth of the bacterium; nor would the latter develop in this raw yeast-water, to which 5 per cent. of cane-sugar was added. The latter fact was thought to be possibly due to the raw yeast-water having inverted the saccharose, and we have seen that glucose and levulose are not suitable media; and we explained similarly the failure of saccharon-Mayer's solution, to which this raw yeast-water was added, as well as failures with brown sugar, and with beet extract similarly made up. That failure should follow with dextrin, with maltose, with levulose, milk-sugar, and with beer wort similarly made up, was only to be expected from experience with these media concocted with boiled yeast-water.

Further experiments, however, led to the conviction that matters were more complicated than would be implied by this explanation.

At one stage in the investigation, being impressed by the stimulus to growth afforded by the addition of (boiled) yeast-water to the saccharose solutions, we tried the effect of adding sugar to the raw yeast extract and sterilising by filtration through porcelain only, and were surprised to find that no growth whatever occurred at any temperature, *e.g.*, 18°, 23°, and 30° C. This was afterwards explained as above—the yeast extract inverts the sugar before it has time to filter.

We then tried a series of experiments as follows:—Cultures at 17—20°, 25°, and 31° C., were made in 10 per cent. saccharon + 10 per cent. yeast-water mixed raw and sterilised by filtration only; no traces of growth occurred in 10 days. The same failure was realised with 10 per cent. saccharon alone sterilised by filtration only; with yeast extract only sterilised by filtration only; and with yeast extract sterilised by filtration and then boiled before adding to the filtered sterile 10 per cent. saccharon solution. In no case did any sign of growth occur.

It is therefore clear that the filter either holds back some body necessary for the nutrition of the bacterium, or destroys it in its passage through the pores. Also that raw yeast extract in some way spoils the

sugar (saccharose) as a food material, probably by inverting it. And nevertheless the bacterium flourishes in conjunction with the living yeast in saccharose solutions. Here is a puzzle which we have not succeeded in explaining.

It may be merely noted that numerous trials were made in other media than those mentioned, among which glycerine and yeast extract, alcohol with saccharon and yeast extract, starch treated with diastase, also potato, carrot, and milk are the most important. No growth of significance was obtained in any case, and the results may be neglected.

The Acidity of the Cultures.

Several tests showed that the cultures of the bacterium are acid, and the following experiments were made. Sterilised blocks of marble were placed in the culture tubes before they were steamed, and then the infections made as before. In saccharose yeast extract, the active growth which resulted was accompanied by a more diffused viscosity than before, and gas bubbles (CO_2) ascended for days. Tubes of mixed dextrose and levulose with yeast extract, treated exactly similarly, became turbid, and gave off bubbles, but no trace of viscosity resulted; the abundant flocculent deposit consisted entirely of the non-sheathed form of the bacterium.

As will appear later, the acid which causes this liberation of CO_2 is mainly acetic acid.

On the Nature of the Gelatinous Matrix or Slime.

A number of experiments were made to determine the nature of the viscous slime and the jelly-like matrix holding the bacteria. Although reasons have already been given for concluding that this is really nothing more than the swollen cell-walls or sheaths investing the bacteria, the possibility of the opalescence and viscosity of the saccharose media being due to the direct action on the sugar of some enzyme or other body excreted by the organism requires investigation, for it is conceivable that such slimes might arise in any of three ways.

(1) As products of metabolism from the interior of the cells, such as certainly occur in the glands of higher plants, *e.g.*, the mucilage hairs of ferns.*

(2) As products of the action outside the cells of some enzyme-like body which escapes from the organism and acts directly on the sugar.†

(3) As products of conversion of the cell-walls of the organism, these swelling up and becoming diffluent as in the case of the ginger-beer plant.‡

* See Gardiner and Ito, 'Annals of Botany,' vol. 1, p. 27.

† See Ritsert, 'Cent. f. Bakt.,' vol. 11, p. 830.

‡ 'Phil. Trans.,' B, 1892, *loc. cit.*

It seemed impossible to test the first suggestion on such minute cells, but attempts were made to test the second one by the following experiment:—

The bacterium was grown in saccharose yeast extract inside a porcelain filter plunged into the same solution; the gelatinous matrix was formed in abundance *inside* the filter, but none was developed outside although the liquids communicated freely through the pores of the filter. Of course the reply may be made that an enzyme of this nature may be unable to traverse the fine pores, and the question must be regarded as still open.

These gelatinous sheaths or "capsules" are now known in many Schizomycetes, among the best examples being that of *B. vermiforme** and *Leuconostoc*,† and it is now pretty generally agreed that these sheaths are composed of dextran,‡ and Liesenberg and Zopf§ made a curious observation with reference to its formation; they found that the addition of calcium chloride favoured the development of the sheath.

Calcium Chloride.

Zopf's paper suggested that we should test the effect of $CaCl_2$, and accordingly solutions of Liebig's extract, peptone-saccharon, and $CaCl_2$ were tried.

In the slightly alkaline liquid at 32° a mere shimmering turbidity was observed in twenty-four hours, and in four days a dense gelatinous clot formed below the turbid liquid.

At 23° the alkaline liquid showed similar turbidity on the second day, and had formed very little gelatinous deposit in four days. In a week, however, it resembled that at 32° C.

The same solution slightly acidulated with a drop of HCl was slightly more turbid in twenty-four hours, but very little of the jelly formed even in a week.

At the end of the week both set of tubes were distinctly acid, and evidently the formation of the jelly is favoured by the slight alkalinity of $CaCl_2$.

This seemed to strengthen the supposition that our bacterium may be the same as Van Tieghem's *Leuconostoc*, but a close comparison does not bear out this view.

It is nevertheless interesting to observe that *Leuconostoc* inverts saccharose, and only forms sheaths in presence of that sugar or of grape-sugar; that these sheaths are explained as the swollen cell-walls, and many other features exist in common with our form.

* Marshall Ward, 'Phil. Trans.,' B, 1892, *loc. cit.*

† Van Tieghem, 'Ann. des Sci. Nat.,' 6th Series, Bot., vol. 4, 1878.

‡ Scheibler, 'Vereinzeitsch. f. Rübenzucker Ind.,' (1874), p. 24.

§ 'Beitr. z. Phys. u. Morph. niederer Organismen,' Heft 1, 1892, p. 1.

The differences may be important in various degrees. *Leuconostoc* appears smaller and shorter, and withstands high temperatures; it succeeds well in grape-sugar; its characters on gelatine media appear to be different; it can be cultivated in milk, and it forms lactic acid in sugar solutions.

How far the differences can be insisted upon cannot be determined until both organisms have been tested side by side.

There is one further observation to be made in support of our contention that the viscosity depends on the deliquescence of the swollen cell-walls. On agar media, which exude water, the growths are slimy rather than gelatinous, and the longer the gelatine cultures are kept, provided they are not allowed to evaporate, the more diffluent the gelatinous lumps become. In liquid cultures, moreover, the gelatinous clot does not spread evenly through the liquid, but remains around the motionless organism.

Mixed Cultures.

Several attempts were made to obtain the typical clumps of the compound organism by infecting tubes of yeast extract and saccharon and other media with both the bacterium and the yeast separately cultivated pure. The success was only partial, however, though it was not difficult to obtain a viscous clot at the bottom of the tubes in which both bacteria and yeast were embedded, the typical stiff jelly clumps floating in the liquid were not formed, and here again we must conclude that some definite proportion of each is necessary.

On the Chemical Changes incident to the Fermentations.

The jelly-like masses of which the organism consisted set up a very vigorous fermentation in beer wort and in solutions of various sugars. Careful cultures showed, as already stated, that the jelly was composed essentially of a bacterium associated with a yeast, and a long series of our experiments has been directed towards ascertaining what part each played in the fermentation, and how far they assisted or impeded each other.

In the greater number of these experiments five flasks were used, each of about 250 c.c. capacity. 150 c.c. of the culture fluid were placed in each, and four of them were sown with pure cultures: (α) of the yeast alone; (β) of the bacterium alone; (γ) of the yeast and the bacterium separately; (δ) of the two in their ordinary condition of association, forming a lump of the jelly. The fourth flask contained only the culture-fluid with no organism of either kind.

The culture-fluids were usually a 10 per cent. solution of some particular sugar to which 10 per cent. of its volume of yeast-water had

been added to furnish the necessary combined nitrogen for the growth of the organism. The yeast-water was prepared by boiling yeast in water for several minutes, and then filtering and sterilising. In one experiment an ordinary beer wort was used without addition of more nitrogenous matter.

The liquids were carefully and repeatedly sterilised in the flasks before the organisms were added.

The fermentations were conducted at a temperature of about 20° C. The marked feature of the fermentation set up by the conjoint organism was the production of a considerable acidity, the liquid after a few days having the appearance and flavour of lemonade. The acidity proved to be due to acetic and succinic acids.

As considerable differences of behaviour were soon manifested, we subjoin the results of typical fermentations.

	Alcohol. Per cent.	Acetic acid. Per cent.	Succinic acid. Per cent.
I. Beer wort, 150 c.c.:—			
The yeast produced	3·5	0·0124	0·068
The bacterium produced	0	0·1734	0·3
II. Cane sugar, 150 c.c. of 10 per cent. solution containing 15 c.c. yeast water:—			
Yeast produced.....	5·0	0·01	0·057
Bacterium produced	0	0·7	0·57
Yeast + bacterium (in the form of the conjoint organism) produced.....	5·0	0·048	0·078
III. Dark brown sugar (mixture of cane sugar and levulose), proportions as in II:—			
Yeast produced.....	4·75	0·026	0·137
Bacterium produced	0	0·596	0·416
Yeast + bacterium separately sown produced	4·0	0·124	0·1
Conjoint organism produced ...	3·7	0·306	0·168
IV. Grape sugar, proportions as in II:—			
Yeast produced	2·2	0·013	0·049
Bacterium produced.....	0	0·012	0·018
Yeast + bacterium produced ...	2·0	0·02	0·097
Conjoint organism produced ...	2·0	0·15	0·046

The formation of the alcohol was thus shown to be due exclusively to the yeast, and in its production the influence of the bacterium was

not manifested. The yeast was found to be capable of fermenting both glucose and fructose (levulose), but to be more active in the presence than the absence of the latter. Only half the amount of alcohol was formed when the fructose was excluded. The latter sugar was more favourable also to the acid fermentation by the yeast. If we compare Experiments II and III, we find that while the same quantity of alcohol was formed in both cases, the proportion of both acetic and succinic acids was about doubled in the presence of fructose.

The bacterium, however, was responsible for the greater amount of the acid formation. While it caused the production of both acetic and succinic acids in greater proportion than the yeast, it yielded far more relatively of the former. In the experiment with beer wort it produced fifteen times as much acetic acid as the yeast; it only gave rise to four to five times as much succinic. With cane-sugar solution it formed seventy times as much acetic and ten times as much succinic acid as the yeast. The same result was obtained with cane-sugar and fructose.

With grape-sugar the bacterium, like the yeast, could do but little. It produced about the same amount of acetic acid, but scarcely more than one-third as much succinic.

The experiments on the fermentations confirm the view expressed in the early portion of the paper, that cane-sugar is the most favourable medium for the bacterium, but before it is of use to it, it undergoes inversion.

The association of the two organisms together introduced a somewhat curious feature of the fermentation. The yeast continued to produce alcohol in the same proportion as when alone, but the acid fermentations were modified considerably. Comparative experiments were made with cane-sugar, dark brown sugar containing fructose, and grape-sugar. In the first two cases the amount of both acetic and succinic acids produced by the conjoint organism was distinctly less than that which was formed by the bacterium alone. In the grape-sugar fermentation the contrary was the case.

The association of the two organisms into the jelly-like clumps was not without its effect upon the progress of the fermentation, and this effect again appeared in connection with the process of the acidification.

With the dark brown sugar the conjoint organism produced twice as much acetic acid as the two separate constituents working together, but only one and a half times as much succinic. With grape-sugar there was a diminution of the succinic acid, which fell to one-half the quantity produced by the yeast and the bacterium, while not in such close association. The acetic acid, on the other hand, increased. In the first two cases again, the presence of the yeast was very considerably inhibitory of the activity of the bacterium, the latter producing far more acid of both kinds when it was alone in the fermenting liquid.

One or two features of the fermentations call for comment. The progress of the fermentation in an acid liquid was so great as to suggest that an enzyme was excreted by the organism, but careful search proved that this was not the case. Both the conjoint organism and its two constituents are normally aërobic, but in all cases the fermentations will proceed, though with slightly less vigour, in an atmosphere of CO_2. The decomposition effected by the bacterium is accompanied by only a very slow evolution of this gas, not greater indeed than would be due to its respiration. In one experiment which was carried on for two months, there was an output of 0·005 gram of CO_2 per day, the gas being absorbed by caustic potash as it was given off, and the fermentation being consequently aërobic.

The intimate association of the two organisms in the lumps of jelly suggested a symbiotic relationship. Experiments made to ascertain how the two affected each other failed however to bear out this view. The influence of the bacterium we have seen to be largely shown in the formation of relatively considerable quantities of both acetic and succinic acids. Cultivations of the yeast were consequently made in the presence of different proportions of acid, with the expectation of finding that an acid medium would be advantageous to it.

Three flasks were prepared, each containing 90 c.c. of 10 per cent. solution of cane-sugar to which 10 c.c. of yeast-water were added. These were then carefully sterilised. To flask A, acetic acid was added till 0·1 per cent. was present; B contained 0·25 per cent., and C 0·5 per cent. of the same acid. Each was then infected with 1 c.c. of a pure culture of the yeast, and they were allowed to ferment at the laboratory temperature (about 10° C.). A control was prepared in a fourth flask, no acid being added. The fermentation which resulted in A was about equal to that in the control; that in B was less vigorous, and that in C was very feeble. After two days, while the fermentation was still active, they were all filtered on to tared filters, and the quantity of yeast weighed. The control was then found to contain 0·09 gram of yeast, while the other flasks contained respectively—A, 0·106, B, 0·079, and C, 0·027 gram. So far as the growth of the yeast was concerned, 0·1 per cent. of acetic acid was favourable; but 0·25 per cent. was slightly inhibitory, while 0·5 per cent. was markedly so.

The alcohol was then distilled off and estimated. A contained 2 per cent., B 1·33 per cent., and C 1 per cent., while the control contained 1·66 per cent. of the spirit. These figures agree with the conclusion based upon the weight of the yeast. The same results were obtained when lactic acid was substituted for acetic.

As in the original experiments made with cane-sugar, the bacterium produced more acetic acid than the highest proportion used in these fermentations, it is clear that the bacterium does not conduce in this way to any increase of growth or activity of the yeast.

It seemed possible that the bacterium might assist the latter by fixing nitrogen from the air, as bacteria have been found to do in other cases of symbiosis. Careful experiments showed, however, that neither the conjoint organism nor the bacterium alone could grow in a culture fluid which did not contain combined nitrogen. A quantitative estimation of nitrogen was made of two fermentations of cane-sugar, one by yeast alone, the other by the conjoint organism, a control of the culture fluid alone being examined simultaneously. The nitrogen was determined by Kjeldahl's process. The initial amount of nitrogen present was 0·013 gram. Neither flask showed any variation from this quantity at the end of the fermentation.

The presence of the bacterium was seen consequently to be of no service to the yeast, but on the other hand to be disadvantageous to its growth.

The yeast was, on the other hand, found to be of some value to the bacterium. During the fermentations the former was found to excrete a certain amount of various extractives into the liquid, which had a distinctly nutritive value to the latter. The bacterium grew very much better at the expense of these extractives than it did when supplied with combined nitrogen in the form of ammonium tartrate, or of asparagin. Comparative experiments made with the extractives prepared from a fermentation showed that neither asparagin nor an ammonium salt could minister to its development so readily as they did. This view was also supported by the fact that yeast-water had already been found to be the best form of supplying combined nitrogen artificially to cultures of the isolated bacterium.

The alcohol was of no nutritive value to the latter. When it was cultivated in the presence of varying quantities of the spirit, it made no use of it, and the original quantity of alcohol was found to be present in the liquid at the conclusion of the experiment.

The relationship between the two is not therefore one of symbiosis. The bacterium appears to be a saprophyte, thriving at the expense of the nitrogenous excreta of the yeast. It is not at all parasitic on its neighbour, the yeast never being injured by its presence until sufficient acid has been produced to cause a secondary inhibition.

In the formation of the acetic acid this bacterium is peculiar. It cannot be referred to the ordinary group of acetifying organisms,* as it has not the power of affecting alcohol as they do. Its action on the sugar seems to be a direct one, causing a formation of acid at the expense of the latter, without setting up any preliminary fermentation. *Bacterium aceti* (Brown) has also this power in the presence of oxygen, but it can oxidise alcohol in addition.

The immediate antecedent of the acid appears to be fructose.

* See Beijerinck, 'Cent. f. Bakt.,' vol. 4, 1898, for a summary of the known acetic organisms.

Though the bacterium flourishes in solutions of cane-sugar, it does not convert the latter immediately into the acids of the fermentation, but hydrolysis takes place first. In one experiment 150 c.c. of a 10 per cent. solution of cane-sugar, containing 15 c.c. of yeast water, were infected in a sterile flask with a pure culture of the bacterium, and fermentation was allowed to proceed for twelve days at the temperature of the laboratory. At the end of that time the acetic acid was distilled off, and the residue poured into alcohol to precipitate another constituent, which will be referred to below. The remainder of the sugar was left in solution in the alcohol. After filtration the alcoholic liquid was evaporated to a syrupy consistency, and the residue taken up with water. It was then divided into two equal portions, and one of them was acidified with sulphuric acid till the concentration of the latter was 2 per cent. It was then boiled on a water-bath for two hours and carefully neutralised. An aliquot part of each was then titrated with Fehling's solution, and the cupric oxide formed was weighed. The weights of the two were almost identical, differing by only 0·002 gram. The inversion of the cane-sugar by the organism had consequently been practically complete.

A few bacteria only have been found to secrete invertase. Fermi and Montesano found that *Bacillus megatherium*, *B. fluorescens liquefaciens*, the red Kiel bacillus, and *Proteus vulgaris* were capable of producing it in bouillon to which cane-sugar had been added. Van Tieghem showed that it was secreted by *Leuconostoc mesentenoides*. To these the bacterium under observation must now be added. The following experiment shows that the inversion noticed in the last case was due to an excreted enzyme.

A good culture was made in cane-sugar solution, and, after a few days, was filtered under pressure through a Chamberlandt's porcelain tube. Three flasks were taken, and 15 c.c. of cane-sugar solution of 2 per cent. concentration placed in each. They were then carefully sterilised. To A and B, 2 c.c. of the filtrate from the culture were added, the filtrate having been found free from organisms by careful microscopic examination; B was then boiled and allowed to cool slowly. They were all kept for a few days at the temperature of 22° C. in an incubator. On titration with Fehling's solution, C, the control, gave no reduction, B a slight one, and A threw down a considerable precipitate of cuprous oxide. The small reduction in B was due no doubt to a trace of invert-sugar present in the culture fluid added to the flask. The much greater reduction in A was due to invertase which the bacteria had excreted.

The experiments quoted in the early part of this paper show, however, that the inversion must take place gradually and the fructose be supplied almost as it is wanted. In presence of fructose only, growth is almost impossible.

Attention has been called in the first part of this paper to the amount of viscous material which the bacterium produces in solutions of cane sugar. There is a great similarity between its action in this respect and that of Van Tieghem's *Leuconostoc*.

An examination was made of the viscous material, a special culture being made in a large flask for the purpose. The slimy material was found to be slowly soluble in water, yielding an opalescent solution. When poured into an excess of alcohol, it gave a bulky flocculent precipitate. This was allowed to settle and the alcohol was decanted, and the wet precipitate thrown on to a filter. When all the spirit had drained away, it was taken from the filter and stirred with water in a beaker. A considerable quantity dissolved, but a good deal of residue was left in suspension. The watery solution was filtered off and examined.

It gave a reddish-purple colour on the addition of iodine, but had no action on Fehling's fluid. Heated with 2 per cent. of sulphuric acid on a water-bath for two hours and then neutralised, it gave evidence of the presence of sugar. It reduced Fehling's fluid on boiling, and yielded an osazone when treated with phenylhydrazine acetate. It deflected a ray of polarised light, and had a specific rotatory power of $[\alpha]_D = +130$.

The residue, which was insoluble in water, was coloured violet on the addition of iodine. It had no action on Fehling's solution, but was converted into a reducing sugar by boiling it with 2 per cent. of sulphuric acid. It was readily soluble in a 10 per cent. solution of caustic soda, and less freely in a 1 per cent. solution. Neutralisation or dilution did not cause it to be reprecipitated. The solution had no action on polarised light.

There were thus found to be present in the viscous liquids two distinct carbohydrates, which possessed much in common with Scheibler's *dextran*, but which were not quite identical with the latter. They both appeared to be members of the hemi-celluloses.

The exact relation of these bodies to the bacterium has not been determined. While there are some grounds for thinking that they are not so much products of fermentation in the strict sense as of its ordinary biological processes, being perhaps only the substance of the different sheaths to which allusion has been made in the first part of this paper, it is not certain that they may not be regarded as products produced altogether outside the organism, in consequence of an alteration of the fructose produced by the hydrolysis. It is altogether unlikely that they resulted from the glucose moiety of the inversion, as this sugar is not a favourable medium for the growth of the organism, and such cultivations as are possible in solutions of glucose do not show the presence of any viscous material.